Passive Immunization of Chickens against *Eimeria maxima* Infection with a Monoclonal Antibody Developed against a Gametocyte Antigen

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Eimeria maxima gametocytes contain two major antigens with molecular masses of 56 and 82 kilodaltons (kDa) which are recognized by convalescent sera from immune chickens. Preparations enriched in these two antigens were used to immunize mice, and several monoclonal antibodies which specifically reacted with the 56-kDa antigen were produced. One of these monoclonal antibodies of the immunoglobulin M subclass, along with immune chicken sera raised against affinity-purified 56- and 82-kDa antigens, was used to passively immunize chicks. On the basis of the parameter of total oocyst output, it was found that these antibodies provided partial protection (40 to 50% inhibition) against *E. maxima* challenge infections.

Avian coccidial parasites of the genus *Eimeria* go through a complex life cycle in the intestinal mucosa of infected birds. After recovery from an infection with a given eimerian species, chickens are immune to reinfection with oocysts from the same species. Despite a great deal of research into the immunology of coccidial infections, it is still not clear which stages of the life cycle are stimulating this protective immune response.

During the life cycle, eimerian parasites undergo both asexual and sexual stages of development. Several recent studies on the asexual stages of development have shown that, by using a variety of monoclonal antibodies, development can be inhibited in vitro (2) and passive immunity can be conferred in vivo (1). In contrast, little is known about the role in protective immunity of the sexual stages of development, apart from one recent study showing the inhibitory effect of a monoclonal antibody on an *Eimeria tenella* gametocyte antigen in vitro (6).

We have been investigating the sexual stages of *E. maxima* development (microgametocytes and macrogametocytes) in order to study their development at the molecular level as well as to assess the importance of sexual-stage antigens in protective immunity (8, 9, 13). We have shown that two gametocyte proteins with molecular masses of 56 and 82 kilodaltons (kDa) were highly antigenic in a variety of hosts (9, 13) and reacted strongly on a Western blot (immunoblot) with sera from chickens which had recovered from *E. maxima* infections. Since similar sera from recovered chickens were shown by Rose to provide good passive immunity against *E. maxima* (11), we proposed that these two gametocyte antigens alone may be effective in stimulating immunity.

In the present study, monoclonal antibodies were prepared against the 56-kDa antigen. We found that one of these monoclonal antibodies, 1E11-11, was outstanding in its reactivity to the 56-kDa antigen on Western blots. This monoclonal antibody was used to passively immunize chickens during the late stages of an *E. maxima* challenge infection. The results showed that a statistically significant inhi-

bition of oocyst output can be achieved by using a single anti-gametocyte monoclonal antibody.

MATERIALS AND METHODS

Parasites. The Houghton strain of *E. maxima* was used throughout these experiments. The oocysts were passaged in Anak 180 Israeli commercial broiler-breed chickens, and clean sporulated oocysts were prepared as described by Wagenbach et al. (12).

Preparation of gametocyte antigens. Gametocytes were isolated from infected intestinal mucosa removed 132 to 136 hours postinfection with 10,000 sporulated *E. maxima* oocysts as described previously (9). The clean gametocytes were extracted with 0.5% Nonidet P-40 (NP-40) in DEB (10 mM Tris [pH 7.4], 50 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]) for 30 min at room temperature and spun at 1,500 × g for 15 min, and the supernatant was collected. Saturated ammonium sulfate was then added to a concentration of 20%, the mixture was incubated for 15 min on ice, the tubes were spun at 1,500 × g for 15 min, and the supernatant was saved. Saturated ammonium sulfate was then added to a final concentration of 50%, and this mixture was incubated for 15 min on ice and spun at 1,500 × g for 20 min. The pellet (10^6 gametocytes) was dissolved in 1 ml of column running buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 0.05% sodium deoxycholate) and dialyzed at 4°C against three changes of the same buffer.

Gametocyte extract used for the enzyme-linked immunosorbent assay (ELISA) and Western blotting methods was prepared with the detergent sodium deoxycholate, as described previously (13).

Sephadex G-200 column chromatography. The 50% ammonium sulfate cut of the gametocyte NP-40 extract was separated by liquid chromatography on a Sephadex G-200 column. Approximately 50 optical density units at 280 nm of the protein extract was applied to a 250-ml Sephadex G-200 column (30 cm in height, 3 cm in diameter) in column running buffer. Three-milliliter fractions were collected and analyzed by Western blotting as described below. The remainder of

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the fraction was stored at −20°C and used later to immunize mice for the production of hybridomas (described below).

Production of monoclonal antibodies to E. maxima gametocyte antigens. Monoclonal antibodies were produced against E. maxima gametocyte antigens as follows. Mice were immunized with four or five weekly injections intraperitoneally of either 100 μg of crude gametocyte detergent extract, 100 μg of enriched "trough" fractions (i.e., those fractions from the Sephadex G-200 column between the two peaks enriched in the 56- and 82-kDa antigens on the basis of Western blotting [see Fig. 1]), or the 56- and 82-kDa protein bands excised from a polyacrylamide gel on which 0.5 to 1.0 mg of crude gametocyte detergent extract was separated by sodium dodecyl sulfate-gel electrophoresis. The final boost was given 3 to 4 days prior to performing fusions. Immune mouse spleen cells and myeloma nonproducing cells (line X63-Ag 8.653) were fused as previously described (4). Hypoxanthine-aminopterin-thymidine-resistant hybridomas were grown and were screened by ELISA with crude gametocyte detergent extract as antigen (9). Positive reacting clones were screened by Western blotting (described below).

For ascites production, BALB/c mice were injected intraperitoneally with 0.5 ml of Pristane (2,6,20,14-tetramethylpentadecane; Aldrich Chemical Co., Inc., Milwaukee, Wis.), and 2 weeks later 10×10^6 to 15×10^6 hybridoma cells were injected intraperitoneally. Immunoglobulin G (IgG) and IgM antibodies were then prepared from ascitic fluid by the standard ammonium sulfate precipitation procedure. Their immunoglobulin subclass was determined either by using class-specific antisera or by metabolic labeling (5).

Finally, an IgM class monoclonal antibody to a mouse T-cell receptor (2B8) was kindly provided by Z. Ben-Sasson of the Department of Immunology, Hebrew University-Hadassah Medical School. It was also injected into Pristane-primed mice for the production of ascites and used as a control in the passive immunization experiments described below.

Western blotting. Western blotting was carried out with mouse, rabbit, or chicken antibodies as described previously (5). A 1.5-mg amount of crude gametocyte detergent extract was used as antigen on each preparative miniblot. Horseradish peroxidase-linked second antibodies (Sigma Chemical Co., St. Louis, Mo.) were used in the immune detection of mouse and chicken antibodies, while ^{125}I-protein A (Amersham, Buckinghamshire, England) was used for the detection of rabbit antibodies.

Affinity purification of gametocyte antigen by using monoclonal antibody. Monoclonal antibody 1E11-11 was used to affinity purify the 56-kDa antigen from crude gametocyte detergent extracts. Briefly, the monoclonal antibody was bound to CNBr-Sepharose 4B (Sigma) according to the manufacturer's instructions (10 mg of monoclonal antibody per ml of swollen CNBr-Sepharose 4B). The Sepharose beads were then washed in a solution containing phosphate-buffered saline, 1 mM PMSF, and 0.1% Tween 20 (polyoxyethylenesorbitan monolaurate). Gametocyte sodium deoxycholate extract dissolved in the same solution was then passed over the column two or three times, and the column was washed until the A_{280} was below 0.01 unit. The antigens were then eluted in 3.5 M $MgCl_2$ (the volume being the same as the starting bed volume) and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM PMSF. The eluate was lyophilized and stored desiccated at −20°C.

Passive immunization studies. Passive immunization of chicks by using monoclonal or polyclonal antibodies was carried out as follows. Two-week-old ANAK 180 chicks

kept coccidium free were challenged on day 0 with 100 sporulated E. maxima oocysts per os. Starting on day 3 postinfection, antibodies were administered intravenously into the wing vein once daily for 5 days. On days 1 to 4, chicks were allowed unlimited time to feed; on day 5 postinfection, chicks were allowed only 30 minutes in which to feed. This limited feeding regimen (30 min of feeding per day) continued throughout the days of feces collection (days 6 to 9 postinfection) in order to decrease fecal mass and standardize the quantity of feed given to each chick. Feces from individual chicks were collected into trays containing 0.5 liter of 2% potassium dichromate. The samples were then homogenized for 1 min, and oocysts were counted in McMaster counting chambers as described previously (12).

Three of the passive immunization experiments were performed with chicks from laying hens which had been maintained coccidium free and treated with the anticoccidial drug Cygro (American Cyanamid Co., Wayne, N.J.). Hens showing significantly reduced anti-gametocyte antibody titers on Western blots were artificially inseminated, and the chicks were hatched and their sera were also analyzed by Western blotting.

RESULTS

Enrichment for the 56- and 82-kDa E. maxima gametocyte antigens. Gametocyte antigens were separated on a Sephadex G-200 column as described in Materials and Methods. The column fractions were monitored at an optical density of 280 nm and were analyzed by Western blotting with rabbit antiserum to crude gametocyte extracts. The results are shown in Fig. 1. As can be seen, the starting material contained several antigenic protein bands (Fig. 1B, crude extract), a few of which were also present in uninfected chicken intestine extract (data not shown). The analysis of the column fractions (Fig. 1A and B, lanes 20 to 33) showed that several proteins of low molecular mass, along with high-molecular-mass species, appeared in the void volume (fractions 20 to 22), indicating that these antigens had aggregated (similar results were obtained even when fractions were run in 8 M urea or when detergents other than sodium deoxycholate were used). The 56- and 82-kDa gametocyte antigens were found to be present in the fractions on the descending part of the first absorbance peak (fractions 23 and 24) and in the fractions between the two major peaks (trough fractions 25 to 30). Since the trough fractions showed a relatively low A_{280} while retaining very strong reactivity on Western blots, they were pooled and used as a source of enriched antigen for the production of monoclonal antibodies.

Preparation of monoclonal and polyclonal antibodies to the 56- and 82-kDa gametocyte antigens. The crude gametocyte extract, enriched trough fractions, and the 56- and 82-kDa protein bands excised from polyacrylamide gels were all used to immunize mice for the production of monoclonal antibodies. Serum from mice immunized with enriched trough fractions reacted strongly with the 56- and 82-kDa antigens (Fig. 2A), while it reacted very weakly if at all with other gametocyte proteins and uninfected control chicken intestine extract (lane 5).

Several fusions were performed by using mice immunized with the various gametocyte antigen preparations. Hybridomas were screened by ELISA and Western blotting with crude gametocyte detergent extract as antigen. Those hybridomas which reacted specifically on ELISA with gametocyte extracts and which did not react with uninfected
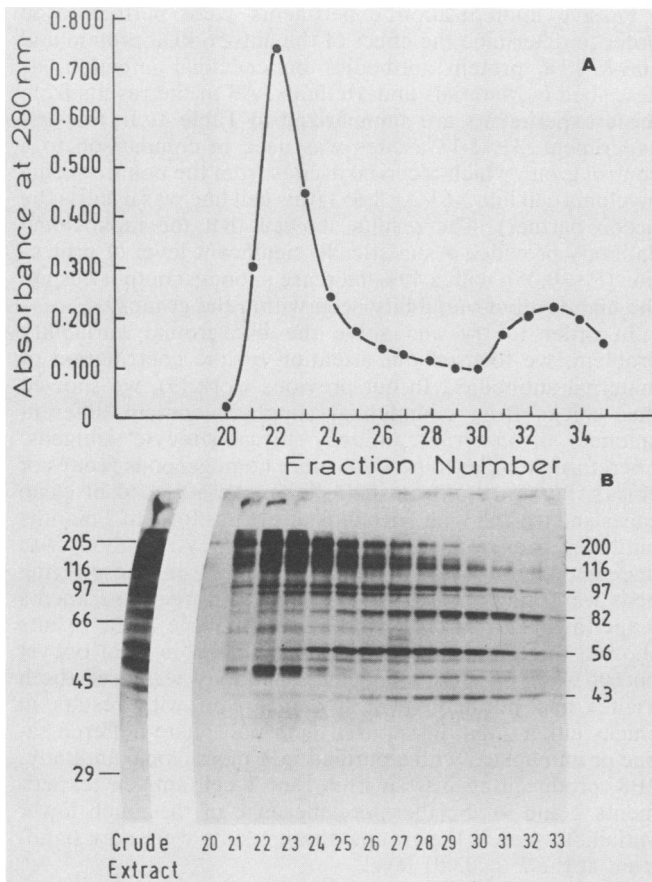
FIG. 1. Separation of antigens from an *E. maxima* detergent extract by Sephadex G-200 column chromatography. (A) An *E. maxima* NP-40 gametocyte extract was prepared as described in Materials and Methods. Fifty optical density units at 280 nm of the extract was applied to a 250-ml Sephadex G-200 column, and 3-ml fractions were collected and monitored at A_{280}. (B) Individual fractions were analyzed by Western blotting using rabbit antiserum prepared against a crude gametocyte detergent extract. The NP-40 extract which was applied to the column is shown in the leftmost lane (crude extract), and the individual fractions are shown in the righthand lanes. Numbers at left correspond to the following molecular mass marker proteins: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase *b* (97 kDa), β-galactosidase from *Escherichia coli* (116 kDa), and myosin (205 kDa). Numbers at right indicate the molecular masses of the major gametocyte antigens.
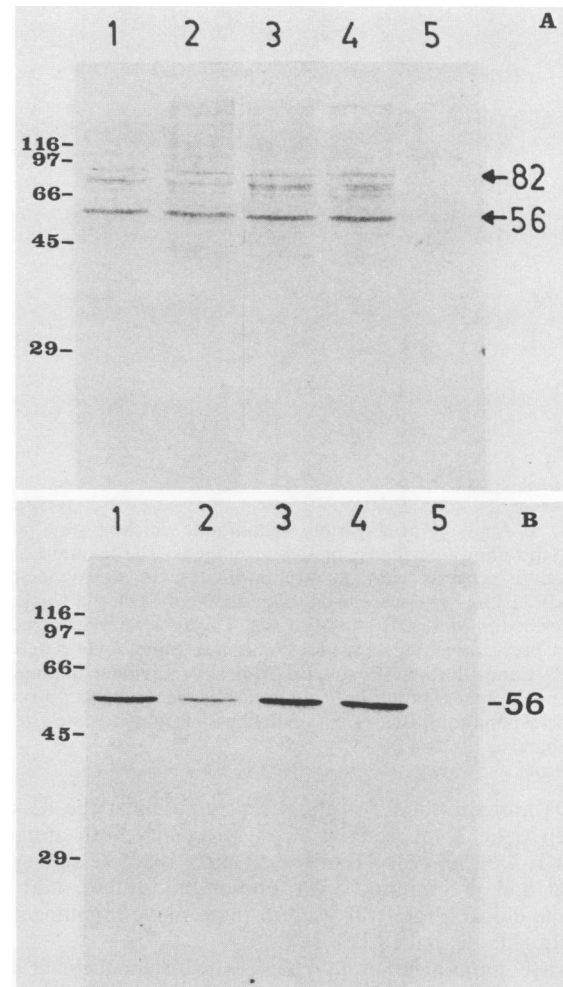

FIG. 2. Immune detection of the 56- and 82-kDa *E. maxima* gametocyte antigens in detergent extracts prepared over the course of a year. Gametocyte detergent extracts prepared over the course of a year were analyzed by Western blotting using either immune mouse sera raised against the trough fractions from the Sephadex G-200 column (A) or monoclonal antibody 1E11-11 (B). A detergent extract prepared from uninfected chicken intestines was used as a control (lanes 5). Numbers at left indicate the molecular mass marker proteins and correspond to those in Fig. 1. Numbers at right indicate the major 56- and 82-kDa protein antigens.

control chicken intestine extract were screened by Western blotting. Most of these hybridomas were found to react with the 56-kDa antigen, while only a few reacted with the 82-kDa antigen. Surprisingly, those hybridomas which reacted with the 82-kDa antigen on initial screening, after further growth and analysis, were found to switch to recognition of the 56-kDa antigen. Furthermore, hybridomas made from mice immunized with the 82-kDa gel piece (and whose sera reacted specifically with the 82-kDa protein band on a Western blot) produced only monoclonal antibodies which reacted with the 56-kDa antigen.

Of the monoclonal antibodies which recognized the 56-kDa antigen, one (1E11-11) was found to be outstanding in its intensity of reaction on Western blots (Fig. 2B). This monoclonal antibody was found to be of the IgM subclass. 1E11-11 bound to Sepharose 4B was used to affinity purify

the 56-kDa antigen, as described in Materials and Methods. Upon Western blotting of the affinity-purified proteins it was found that both the 56-kDa gametocyte protein and the 82-kDa gametocyte protein were present in the eluted material (data not shown).

The affinity-purified antigens were then used to produce highly specific chicken anti-gametocyte serum. Six-week-old chickens maintained coccidium free from the time of hatching were immunized with the purified antigens, and the immune sera were analyzed by Western blotting. As can be seen in Fig. 3, all of the sera tested reacted with both the 56-kDa antigen and the 82-kDa antigen. In addition, two other protein bands of 43 and 35 kDa also appeared in some of the lanes. The 43-kDa protein was previously shown to bind chicken IgG regardless of its specificity (13). The origin of the 35-kDa band is not presently understood; however, it may be the result of protein degradation. Some of the immune chicken sera reacted predominantly against the
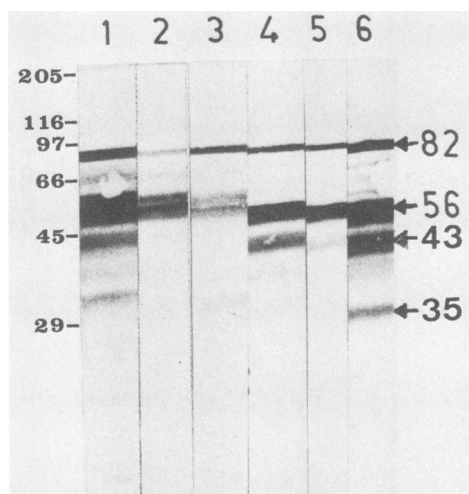
FIG. 3. Analysis of a variety of immune chicken sera raised against the affinity-purified gametocyte antigens. The 56- and 82-kDa gametocyte proteins were affinity purified by using monoclonal antibody 1E11-11 and were used to immunize 6-week-old chickens, as described in Materials and Methods. The sera were analyzed by using a preparative Western blot containing gametocyte detergent extract. Lanes 1 to 6 show sera from six individual immunized chickens. Numbers at left indicate molecular mass marker proteins and correspond to those in Fig. 1. Numbers at right indicate sizes of major bands.

56-kDa antigen (lane 2); others reacted against the 82-kDa antigen (lane 3) or reacted strongly against both antigens (lanes 1, 4, 5, and 6). All of the strongly reactive sera were pooled and precipitated with ammonium sulfate, and the immune chicken IgG was used in the passive immunization experiments described below.

Passive immunization of chickens with monoclonal and polyclonal antibodies. In order to perform passive immunization experiments with the monoclonal and polyclonal antibodies described above, it was important to determine whether the 56- and 82-kDa gametocyte antigens are subject to variation, at least during parasite passage. In order to study antigenic variability, gametocyte extracts prepared and stored over the course of a year were analyzed on Western blots with both immune mouse sera and monoclonal antibody 1E11-11. As can be seen in Fig. 2A and B, little difference was seen in either the size or the intensity of the 56- and 82-kDa protein bands in these gametocyte extracts. Thus, these two proteins appear to be antigenically stable, indicating that antigenic variation may not be a problem in conducting the series of passive immunization experiments described below.

Passive immunization experiments were performed in order to determine the effect of the anti-56-kDa protein and anti-82-kDa protein antibodies on coccidial infection, as described in Materials and Methods. All of the results from these experiments are summarized in Table 1. In the first experiment, 1E11-11 ascites was used in comparison to a control group which received ascites from the nonproducing myeloma cell line X63-Ag 8.653 (this cell line was used as the fusion partner). The results showed that the monoclonal antibody provided a statistically significant level of protection ($P < 0.05$), with a 40% decrease in oocyst output despite the high level of variability seen within the groups.

In order to try and solve the background variability problem, we focused our attention on the contribution of maternal antibodies. In our previous work (5), we showed that chicks from commercial suppliers contain different amounts of maternal antibody to gametocyte antigens. Therefore, in order to produce more homogeneous groups of chicks, laying hens from field flocks were placed in clean cages and treated with coccidiostatic drugs for 2 to 3 months until their background level of anti-gametocyte antibody was undetectable on Western blots. Offspring from these laying hens were then used to conduct the last three experiments (experiments 2 to 4). As can be seen in Table 1, the results showed once again a 40 to 50% reduction in total oocyst output when 1E11-11 monoclonal antibody was used (both ascites and purified IgM) in comparison with results in chicks either sham immunized with phosphate-buffered saline or immunized with a purified IgM monoclonal antibody, 2B8, produced against an irrelevant T-cell antigen (experiments 2 and 3). Furthermore, because of the much lower variability seen in the groups, these results were now significant at the $P < 0.001$ level.

In the last experiment (experiment 4), specific anti-56-kDa protein and anti-82-kDa protein immune chicken IgG was used. There was a 49% inhibition in oocyst output, which is a result similar to the results obtained by using the monoclonal antibody 1E11-11 alone (Table 1). These results indicate that the use of antibodies to both the 56-kDa antigen and the 82-kDa antigen did not significantly improve the protection observed when the anti-56-kDa monoclonal antibody was used alone.

DISCUSSION

Several previous studies had shown that immune sera from chickens repeatedly infected with *E. tenella* and sera from chickens collected 14 days after infection with *E. maxima* provided good passive protection against homologous challenge infections (11). In a recent study by Crane et al. (1), passive protection against *E. tenella* infection was achieved by using a single monoclonal antibody to an *E.*

TABLE 1. Summary of passive immunization results obtained by using monoclonal and polyclonal antibodies

Expt.	Control group[a]	Oocyst counts (10^6)[b]	Experimental group	Oocyst counts (10^6)	% Reduction	Level of significance ($P <$)[c]
1	653 ascites	5.9 ± 5.0	1E11-11 ascites	3.6 ± 2.4	40	0.05
2	PBS	15.1 ± 5.2	1E11-11 IgM[d]	8.7 ± 3.4	43	0.001
3	2B8 IgM	8.4 ± 3.7	1E11-11 IgM	5.1 ± 2.2	39	0.001
4	PBS	2.5 ± 1.9	Immune sera[e]	1.2 ± 1.0	49	0.02

[a] PBS, Phosphate-buffered saline.
[b] Mean ± standard deviation with 20 to 25 chicks per group.
[c] Significance as determined by the Student t test.
[d] IgM of monoclonals prepared by ammonium sulfate precipitation as described in Materials and Methods.
[e] Immune chicken sera prepared against the affinity-purified 56- and 82-kDa antigens.

tenella sporozoite surface antigen. Thus, it appears that antibody could play an important role in protective immunity to eimerian parasites and that defined antigens which elicit the proper humoral immune response may be useful as part of a subunit vaccine for coccidiosis in chickens.

In our previous work, we postulated that the two major *E. maxima* gametocyte antigens, with molecular masses of 56 and 82 kDa, may play an important role in protective immunity (13). In order to more directly study their role in parasite development and immunity, it was of importance to establish methods which would enrich for these two proteins for the purpose of immunizing mice and producing monoclonal antibodies. In the present study, two methods were used to enrich for these gametocyte antigens: Sephadex column chromatography and polyacrylamide gel electrophoresis. By using either method, it was found that mice immunized with enriched 56- or 82-kDa antigen or even with crude gametocyte extracts produced antibodies mainly against these two antigens. This result agrees with our previous work, which showed that sera from mice immunized with whole-gametocyte extracts reacted predominantly with the 56- and 82-kDa antigens (13).

The finding that hybridomas which produced monoclonal antibodies against the 82-kDa antigen were found to switch to recognition of the 56-kDa antigen was very surprising. This result, together with the finding that by affinity chromatography 1E11-11 monoclonal antibody bound both 56- and 82-kDa antigens, indicates either that there are cross-reactive epitopes in these two proteins or that these gametocyte antigens aggregate, as was observed during column chromatography of gametocyte extracts even under reducing conditions (Fig. 1). Further work is required to answer this question.

In the present study, we found a statistically significant level of passive protection by using both polyclonal and monoclonal antibodies to the 56- and 82-kDa *E. maxima* gametocyte antigens. These results indicate that these gametocyte antigens play a role in protective immunity and confirm the results described above, which show that antibody can reach the intestinal mucosa and affect parasite development.

We used the parameter of total oocyst output as a means of assaying the effect of these antibodies. Achieving a reduction in oocyst output is important in terms of preventing the spread of the disease and is analogous to the concept of transmission-blocking immunity in malaria (3). Furthermore, in *E. maxima*, gametocytes appear to be a major cause of pathology. Thus, immunity to the gametocyte stage of development of *E. maxima* may not only effectively block parasite transmission but also act to reduce the pathogenicity of the disease.

The mechanism by which antibodies to the 56- and 82-kDa gametocyte antigens inhibit oocyst output is not currently understood. In a previous study on the biosynthesis of gametocyte proteins (8), we found that these two antigens are detected only when the gametocytes become visible by light microscopy of intestinal smears. This finding, together with the effectiveness of antibodies administered late in the course of infection (days 3 to 7 postinfection in our study and even days 4 to 8, as shown by Rose [11]), indicates that these antibodies are inhibiting either the growth, the development, or the fertilization of the gametocytes. Further work using in vitro systems is required in order to elucidate the precise role of these antigens in gametocyte growth and development.

Chicks from laying hens that had been kept coccidium free for several months showed less variability in their suscepti-

bility to challenge infections with *E. maxima* on the basis of oocyst output. The use of these chicks made it possible to assay more accurately and consistently the effects of the anti-gametocyte antibodies which were tested. These results agree with those of previous studies indicating that maternal antibody plays an important role in protective immunity in young chicks (10).

Antigenic diversity in *E. maxima* is considered to be a major problem in the development of a vaccine against this species (7). This is based on the finding that an infection with one strain of *E. maxima* does not protect against challenge with a different strain. In this regard, it is of importance to note that, at least during passage of the Houghton strain of *E. maxima*, there appeared to be little variability in the 56- and 82-kDa gametocyte antigens. Furthermore, we have also shown that sera taken from Israeli chickens grown under field conditions produce antibodies which strongly react with the 56- and 82-kDa antigens of the Houghton strain of *E. maxima* (13). Once the genes encoding these antigens are cloned and sequenced, their actual degree of strain diversity will be determined. Furthermore, these clones may be useful in identifying homologous genes in other eimerian species. These gene products represent a potential transmission-blocking component of a subunit vaccine for coccidiosis in chickens.

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